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Recovery and Diversity of Heterotrophic Bacteria from Chlorinated Drinking Waters

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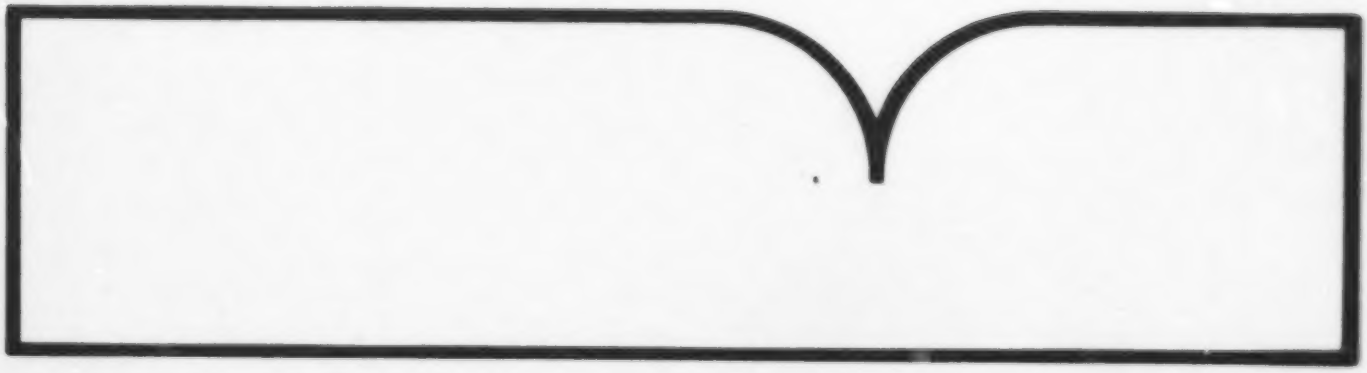

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Project Officer, Donald J. Reasoner
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The standard plate count (SPC) procedure that has been recommended for the enumeration of heterotrophic bacteria involves a pour plate technique with a 2-day incubation at 35°C (1). However, numerous investigations have shown that this standard method frequently provides lower counts of bacteria from drinking waters than do other procedures (6, 15, 20).

The objectives of this investigation were twofold: (i) to evaluate alternative procedures for the enumeration of heterotrophic bacteria from chlorinated water supplies and (ii) to develop a procedure that could be used to identify the bacteria that commonly occur in drinking waters.

We report here the results of using alternative methods for the enumeration of heterotrophic bacteria. The identification of the bacteria that occurred in the chlorinated waters that were sampled will be presented elsewhere (S. J. LaCroix, J. S. Maki, D. J. Reasoner, and J. T. Staley, manuscript in preparation). The majority of these were gram-negative, nonfermentative rods, many of which were pigmented yellow to brown. These could not be placed in the poorly defined genus *Flavobacterium* without further tests, such as DNA-DNA hybridization. Known genera identified in this study were *Caulobacter*, *Hyphomonas*, *Aquaspirillum*, *Vibrio*, and *Gluconobacter*.

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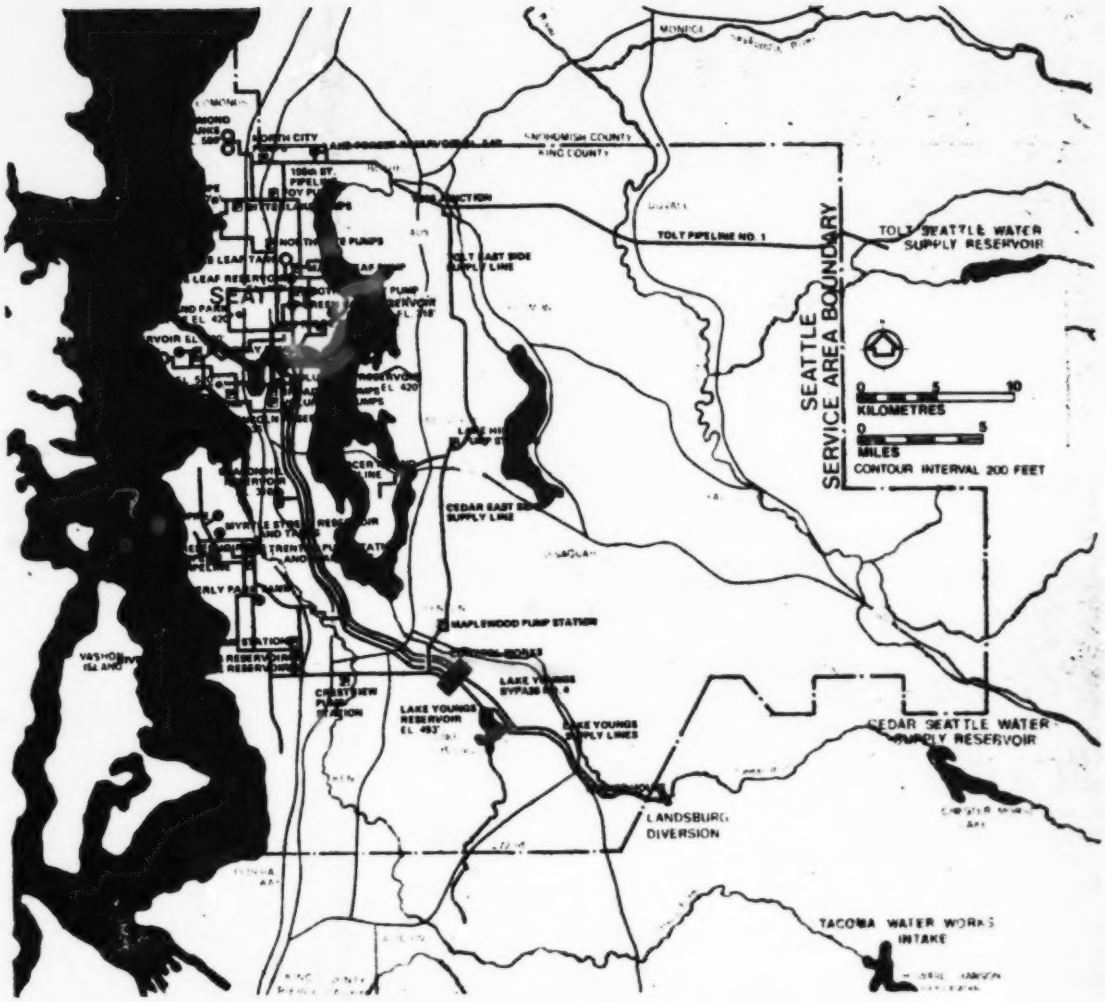

FIG. 1. Map showing the water distribution system of Seattle (courtesy City of Seattle).

tions were made on seven occasions from January 1981 to February 1982 prior to the introduction of the liming process. Sterile Whirlpak bags or Nalgene bottles were used for the collection of viable samples. The containers used for the collection of chlorinated water also held prescribed amounts of sodium thiosulfate to neutralize residual chlorine (1). Residual chlorine measurements were made at the sites at the time of collection by using the *N*,*N*-diethyl-*p*-phenylenediamine method (1) and a Hach test kit (Hach Chemical Co., Ames, Iowa). Temperature was recorded at the time of collection, and pH measurements were also made then or after returning to the laboratory by using water samples without added thiosulfate. All chlorinated samples, with the exception of sample D2 (from the Tolt system), were collected from flowthrough taps. Final chlorinated samples were collected at the earliest sample point following the in-city reservoir that would permit a 10-min chlorine contact time at maximum flow rates. Viable samples were held on ice until ready for processing, all of which was completed within 8 h of sample collection.

Four media were used to recover heterotrophic bacteria from the Seattle water distribution system (Table 2). The media were standard methods agar (SMA) (1), R2A (15), casein-peptone-starch agar (CPS) (10), and dilute peptone agar (DP) (18). Spread plates of each medium were made in triplicate at several dilutions, routinely incubated at 20°C, and enumerated after 28 to 30 days. Data reported here are from normal-sized petri plates (100-mm diameter), but larger-sized plates (150-mm diameter) were also used occasionally. SMA was also used in pour plates as previously described (1) and incubated for 48 h at 35°C.

Enumeration studies. Initial investigations involved comparing the mean numbers of CFU recovered on spread plates of the four media inoculated with chlorinated water samples. Plates were incubated at 20°C and enumerated after 28 to 30 days. The mean numbers of CFU were analyzed nonparametrically for differences by using the Kruskal-Wallis test (11, 25). Significantly different data ($P < 0.05$ or $P < 0.10$) were further analyzed by using a nonparametric multiple-range test (25). Comparisons of a like manner were also

made of the mean numbers of CFU recovered on spread plates of DP and CPS incubated at 20°C for 28 to 30 days and SMA pour plates incubated at 35°C for 48 h after inoculation with chlorinated water samples. The mean numbers of CFU recovered on spread plates of DP and CPS inoculated with chlorinated water samples and incubated as described above were compared with each other by using the Wilcoxon paired-sample test (25). In two sampling surveys the effect of incubation temperature on the numbers of CFU recovered on spread plates of DP and CPS was examined. After inoculation with chlorinated water samples, spread plates of DP and CPS were incubated at 10, 20, and 30°C and enumerated after 28 to 30 days. Furthermore, some plates were incubated at 20°C for 2 days and then transferred to 30°C for the remainder of the time. Data for each medium were analyzed separately by using the Kruskal-Wallis test (11, 25), and significantly different data ($P < 0.05$ or $P < 0.10$) were further analyzed by using a nonparametric multiple-range test (25).

Effect of physical and chemical factors of the treatment system on the recovery of viable bacteria from treated samples. Statistical analyses were performed to determine whether any of the measured physical and chemical factors of the treatment and distribution process had a reproducible effect on bacterial recovery from chlorinated samples. Thus, residual chlorine concentration, temperature, and pH were included in a linear correlation analysis (25) with the mean numbers of CFU recovered on DP and CPS for samples from the same places and times. The mean numbers of CFU recovered on DP and CPS inoculated with chlorinated samples were also compared with the mean numbers of CFU recovered on the same media inoculated with the immediately preceding reservoir sample (which had no chlorine residual), expressed as a percentage of CFU surviving chlorination, and normalized by using an arc sine transformation. These normalized percentages were also compared with chlorine concentration, temperature, and pH by using a linear correlation analysis (25).

The mean numbers of CFU recovered on DP and CPS inoculated with chlorinated samples and the normalized percentages were also used in multiple regression analyses (25) versus the following combinations: chlorine plus temperature plus pH; chlorine plus temperature; chlorine plus pH; and temperature plus pH.

Diversity measurements. Strain diversity was determined by examination of the plate(s) of the medium that provided the highest mean numbers of CFU. A given number of colonies (usually 35 to 50 in total) from a given sample on a particular date were analyzed. If the plate(s) contained too many colonies, excess colonies were deleted by random sectoring of the plate. The colonies were carefully examined with a dissecting microscope, and each type noted was considered to be an individual strain. Thus, the colony type was used as an indicator of the strain type (6, 17). The Shannon index was used as an estimate of strain diversity (16, 17).

Direct AO microscopic counts. For direct acridine orange

TABLE 1. Sample locations selected in the Cedar River portion of the Seattle water distribution system

Sample location	Code	Description
Landsburg	CPR1	Prior to chlorination
Landsburg	CPT0	After first chlorination
Lake Youngs	LY	
Lake Youngs after chlorination	CLT2	After second chlorination
Lincoln Reservoir	Res. I3	
Lincoln Reservoir	I3	After third chlorination
Volunteer Park Reservoir	Res. K3	
Volunteer Park Reservoir	K3	After third chlorination
West Seattle Reservoir	Res. L3	
West Seattle Reservoir	L3	After third chlorination
Myrtle Reservoir	Res. Myr	Water after L3 (i.e., after third chlorination
Myrtle Reservoir	Myr	After fourth clorination
Beacon North Reservoir	Res. M3	
Beacon North Reservoir	M3	After third chlorination
Riverton Heights Reservoir (covered)	P1	After second chlorination

TABLE 2. Formulas and organic contents of the media used in this study

Ingredient	Amt (g · liter^{-1}, unless otherwise indicated) of ingredient in:			
	SMA	R2A	CPS	DP[a]
Peptone (P) or tryptone (T)	5.0 (P)	0.5 (T)	0.5 (P)	0.1 (P)
Yeast extract	2.5	0.5		
Glucose	1.0	0.5		
Casamino Acids (CA) or sodium caseinate (SC)		0.5 (CA)	0.5 (SC)	
Starch (soluble)		0.5	0.5	
Glycerol			1.0 ml	
Sodium pyruvate		0.3		
K_2HPO_4		0.3	0.2	
$MgSO_4 \cdot 7H_2O$		0.05	0.05	0.6
$FeCl_3$			Tr	0.02
Vitamins				Tr
Total amt (g · liter^{-1}) of organic ingredients (excluding agar, chelating agents, and vitamins)	8.5	2.8	2.5	0.1
Amt (mg · liter^{-1}) of total organic carbon[b] (excluding agar, chelating agents, and vitamins)	3,170.0	ND[c]	733.0	77.3

[a] Also contains other inorganic compounds and organic chelating agents in a trace elements solution (18).

[b] Determined by using a Sybron/Barnstead Photochem organic carbon analyzer.

[c] ND, Not determined.

TABLE 3. Comparisons of the mean numbers of CFU recovered on spread plates of the four media inoculated with chlorinated samples

Date	Sample	P^a	Result	Chlorine (mg · liter^{-1})
1/7/81	K3	<0.05	DP = CPS > SMA > R2A	0.20
1/7/81	P1	<0.10	SMA = DP > CPS = R2A	0.50
1/28/81	D2	<0.05	CPS = DP > R2A = SMA	Tr
3/19/81	I3	<0.10	CPS = DP > SMA = R2A	0.44
3/19/81	K3	<0.10	DP = R2A = CPS > SMA	0.42
3/19/81	L3	<0.10	DP > CPS = R2A = SMA	0.40
3/19/81	M3	<0.10	DP > R2A = SMA = CPS	0.50
3/19/81	P1	<0.10	DP > R2A = SMA = CPS	0.60

[a] The differences shown were determined with a nonparametric multiple-range test ($P < 0.05$ or $P > 0.10$) after significant differences were found with the Kruskal-Wallis test ($P < 0.05$ or $P < 0.10$).

(AO) microscopic counts, samples were fixed with formaldehyde to a final concentration of 1% at the time of collection. The staining procedure of Hobbie et al. (9) was used to determine the number of bacteria per milliliter. A Zeiss GFL microscope with an epifluorescence attachment was used to visualize stained cells (19).

RESULTS

Recovery studies. The first enumeration studies involved samples of chlorinated drinking waters derived from reservoirs that received water from the Tolt River, the Cedar River, or combined river sources. Comparison of the mean numbers of CFU recovered on the four media at 12 stations revealed significant differences at 8 of the stations ($P < 0.05$ or $P < 0.10$, Kruskal-Wallis test). Further analyses with the nonparametric multiple-range test are shown in Table 3. Results indicated that DP generally recovered the highest mean numbers of CFU. CPS also recovered high mean numbers of CFU (the range of CFU recovered on CPS was 3 to 164% of the CFU recovered on DP, and the range of CFU recovered on R2A was 10 to 120% of the CFU recovered on DP), so further analyses were conducted with these two media. R2A, which is similar in composition to CPS, was found to be comparable to CPS at most sites, a result which is consistent with previous results from this laboratory (6).

The mean numbers of CFU recovered on spread plates of DP and CPS incubated for 28 to 30 days at 20°C were compared with the mean numbers of CFU recovered on pour plates of SMA incubated at 35°C for 48 h (SPC procedure) ($n = 19$). In 57% of the 19 samples no colonies were observed on the SMA pour plates, and the mean numbers of CFU recovered ranged from <3.0 to 2.2×10^5 CFU · ml^{-1} on DP and from 1.0×10^1 to 1.7×10^5 CFU · ml^{-1} on CPS (Table 4). For the remaining samples of which CFU were found on the SMA pour plates (Fig. 2), significant differences were observed in five of the eight samples ($P < 0.05$ or $P < 0.10$, Kruskal-Wallis test). For I3, K3, and P1 samples collected on 7 January 1981 (1/7/81), the mean numbers of CFU recovered on DP and CPS were significantly different from those recovered on SMA ($P < 0.05$, nonparametric multiple-range test). For I3 samples collected on 11/12/81 and CPT0 samples collected on 2/24/82, the nonparametric multiple-range test could not be used because of unequal numbers of datum points (25). However, Fig. 2 shows that for the samples from these two stations, the mean numbers of CFU recovered on both DP and CPS were higher than those recovered on SMA.

For Cedar River distribution system samples, the mean numbers of CFU recovered on DP were compared with the mean numbers of CFU recovered on CPS by using the Wilcoxon paired-sample test. This was done to compare recoveries on both media to determine if recoveries on one were consistently higher than those on the other. Data were included only when the number of replica plates of each medium was three ($n = 19$). DP was found to provide significantly higher mean numbers of CFU when paired samples were compared ($P < 0.05$, Wilcoxon paired-sample test).

TABLE 4. Mean numbers of CFU (±standard deviation) recovered by the SPC procedure (no colonies observed) and on spread plates of DP and CPS incubated at 20°C for 28 to 30 days[a]

Date	Sample	Mean CFU · ml^{-1} (±SD) recovered by or on:			Residual chlorine (mg · liter^{-1})
		SPC	DP	CPS	
7/8/81	I3	<0.3	$2.20\ (0.42) \times 10^5$	$1.70\ (0.32) \times 10^5$	0.70
9/2/81	I3	<0.3	$5.10\ (0.95) \times 10^2$	$2.30\ (1.55) \times 10^1$	ND[b]
9/2/81	K3	<0.3	$1.70\ (0.26) \times 10^3$	$1.00\ (0.28) \times 10^3$	ND
9/2/81	L3	<0.3	$2.90\ (0.14) \times 10^3$	$3.50\ (1.10) \times 10^3$	ND
9/2/81	M3	<0.3	$1.50\ (0.20) \times 10^5$	$9.60\ (2.05) \times 10^4$	ND
11/12/81	K3	<0.3	$7.00\ (4.25) \times 10^3$	$6.00\ (3.60) \times 10^3$	0.50
2/24/82	I3	<0.3	$4.30\ (0.60) \times 10^3$	$5.60\ (2.10) \times 10^3$	0.80
2/24/82	K3	<0.3	$<3.0 \times 10^1$	$1.30\ (1.10) \times 10^3$	0.70
2/24/82	L3	<0.3	$1.00\ (0.70) \times 10^1$	$1.70\ (0.60) \times 10^3$	0.50
2/24/82	M3	<0.3	$3.00\ (6.00) \times 10^2$	$<3.0 \times 10^2$	0.80
2/24/82	Myr	<0.3	$3.00\ (1.75) \times 10^3$	$1.00\ (1.00) \times 10^3$	0.50

[a] Unless otherwise indicated, results are from triplicate plates.
[b] ND, Not determined.
[c] Results are from duplicate plates.

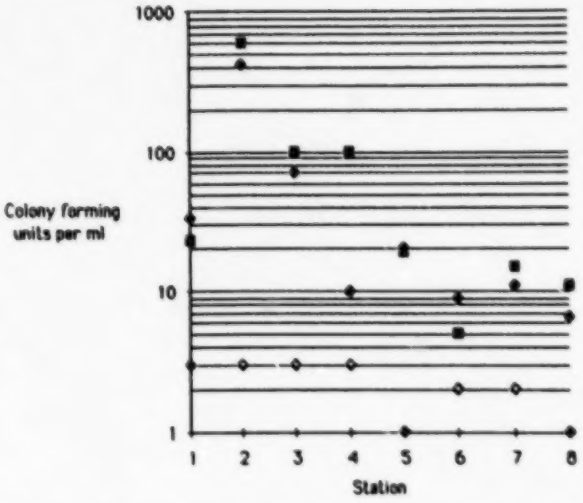

FIG. 2. Semilogarithmic plot of the mean numbers of CFU recovered on SMA pour plates (◇), DP spread plates (■), and CPS spread plates (◆). Data were collected at three separate times: stations 1 to 3 (I3, K3, and P1) on 1/7/81; stations 4 to 6 (CPT0, CLT2, and I3) on 11/12/81; stations 7 and 8 (CPT0 and CLT2) on 2/24/82. Mean counts were based on three plates for all stations and media except for DP at stations 4 and 7 (two plates) and station 4 (one plate) and for CPS at station 6 (two plates) and stations 4 and 5 (one plate). Standard deviations of the means (data not shown) were significantly different (Kruskal-Wallis test) at $P < 0.05$ for stations 1, 2, and 3 and at $P < 0.10$ for stations 6 and 7.

Samples collected on 11/12/81 and 2/24/82 were used to examine the effects of incubation temperature on the recovery of CFU on DP and CPS. On 11/12/81 replicate sets of triplicate plates inoculated with chlorinated water from M3 were incubated for 28 to 30 days at 20 or 30°C or for 2 days at 20°C followed by a transfer to 30°C for the remainder of the time. On 2/24/82 sets of triplicate plates inoculated with chlorinated water from K3, M3, and Myr were incubated similarly. Plates inoculated with chlorinated water from I3 and L3 were incubated as described above, and an additional set was incubated at 11°C.

For M3 samples collected on 11/12/81 (Fig. 3), significant differences between the mean numbers of CFU recovered on DP and CPS were found for the different incubation temperatures ($P < 0.05$, Kruskal-Wallis test). For M3 samples incubated at the different temperatures, the mean numbers of CFU recovered on DP were all significantly different from each other ($P < 0.05$, nonparametric multiple-range test); the mean numbers of CFU recovered on CPS incubated at 20°C were significantly different from those recovered on CPS incubated at other temperatures ($P < 0.05$, nonparametric multiple-range test). No statistical difference between the mean numbers of CFU recovered on DP or CPS incubated at the various temperatures was found for K3, L3, M3, and Myr samples collected on 2/24/82 ($P > 0.05$, Kruskal-Wallis test). Significant differences were observed (Kruskal-Wallis test) for I3 samples for both DP ($P < 0.10$) and CPS ($P < 0.05$). For I3 samples incubated at 20°C (Fig. 3), the mean numbers of CFU recovered on DP were significantly different from all the other means ($P < 0.10$, nonparametric multiple-range test); unequal numbers of datum points prohibited the use of the nonparametric multiple-range test for CPS. Figure 3 indicates that the mean numbers of CFU on plates grown at 20°C were the highest for this station.

Effect of physical and chemical factors of the treatment system on the recovery of viable bacteria from treated samples. A linear correlation analysis of the mean numbers of CFU recovered on DP and CPS inoculated with chlorinated samples with residual chlorine concentration, temperature, and pH did not indicate any significant correlations, either positive or negative. This was also true of the normalized percentage of CFU surviving chlorination on each medium inoculated with the immediately preceding reservoir sample.

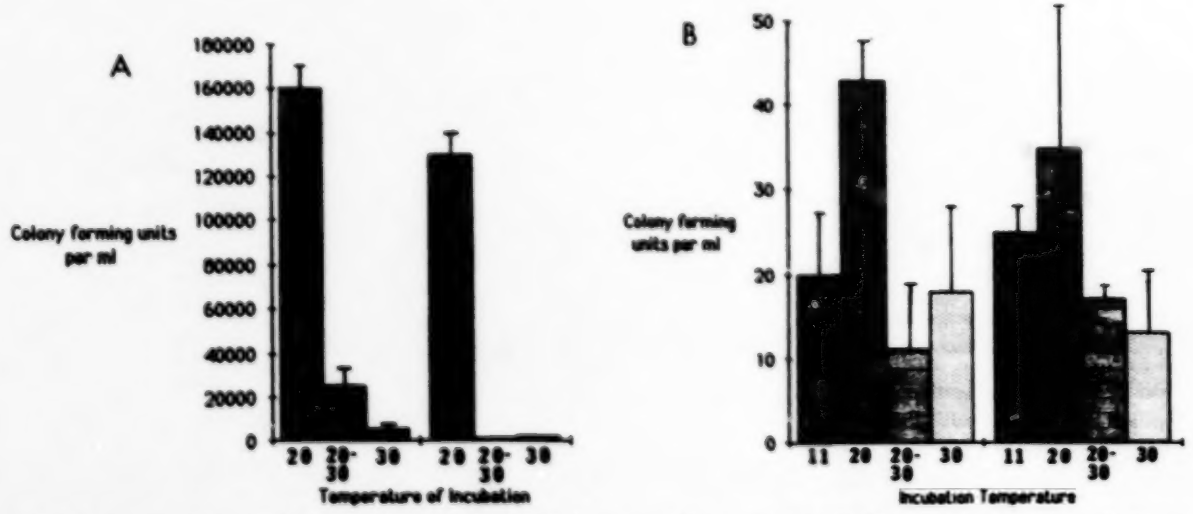

FIG. 3. CFU at different incubation temperatures after 28 to 30 days on spread plates. (A) 11/12/81 M3 samples on DP (left set of columns) and on CPS (right set of columns). (B) 2/24/82 I3 samples on DP (left set of columns) and on CPS (right set of columns). Bars denote the upper limit of one standard deviation of the mean.

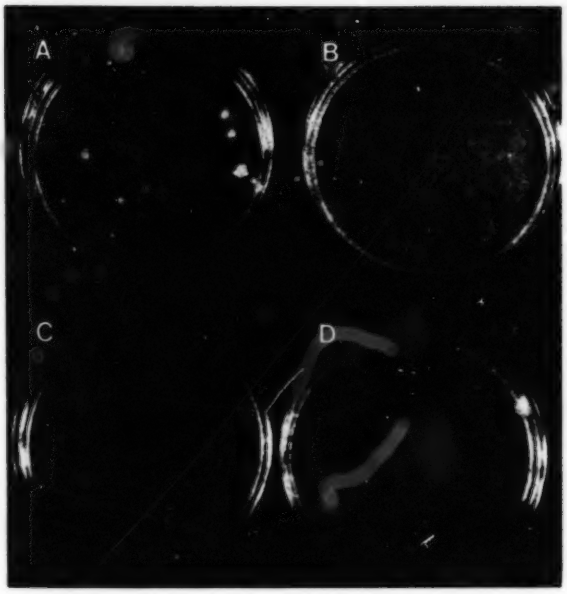

FIG. 4. Four plates showing colony types at various points in the Seattle water distribution system. (A) Cedar River source water (CPR1) prior to the first chlorination (5.5×10^3 bacteria per ml on DP). (B) Lake Youngs (LY) following the first chlorination (1.0×10^5 bacteria per ml on DP). (C) West Seattle reservoir (Res. L3) following the second chlorination (1.5×10^5 bacteria per ml on DP). (D) Tap water sample removed at West Seattle Reservoir (L3) following the third and final chlorinations. Of those illustrated, this is the only sample that had a chlorine residual; it contained 3.1×10^3 bacteria per ml on DP. Note the high diversity of colony types on the source water sample relative to all the downstream samples and the high numbers of bacteria in the reservoir samples relative to the source water sample. Virtually all the colonies found on plates prepared for the chlorinated samples (i.e., LY, Res. L3, and L3) were pigmented yellow, orange, or red.

Multiple regression analyses with the mean numbers of CFU recovered on DP and CPS and the normalized percentages versus the different combinations of parameters did not indicate the presence of any significant relationships.

Diversity. There was always a dramatic difference between the variety of colony types of heterotrophic bacteria growing on plates of source waters as compared with plates of chlorinated waters. Although a large variety of colony types grew on plates inoculated with source water samples, chlorinated water samples appeared to have much less diversity (Fig. 4). Furthermore, unlike the colonies from source water samples, a high proportion of the colonies from chlorinated water samples were pigmented various shades of yellow, brown, or red.

To assess quantitatively whether source waters were more diverse than final chlorinated samples, we took a census of colony types and calculated the diversity index (Table 5). The results confirmed the macroscopic observations. A diversity index of 4.14 was obtained for source waters, whereas final chlorinated samples had diversity indexes ranging from 1.08 to 2.81. In Table 5 the diversity of heterotrophs in source waters is calculated from CPS plates, whereas in the final chlorinated samples the diversity is calculated from DP plates, because these media provided the highest recoveries for their respective sampling sites. Values for three of the final chlorinated samples were increased somewhat with the appearance of a slow-growing *Hyphomonas* sp. that occurred in some of the samples. Thus, after 40 days the diversity index of I3 samples increased to 2.45, that of L3 samples increased to 1.45, and that of M3 samples increased to 1.55.

It was obvious simply by examination of the plates that the reduction in diversity appeared in reservoirs even after the first chlorination (Fig. 4). LY and all subsequent stations sampled showed reductions in diversity in comparison with source waters (CPR1 samples).

Direct counts. Direct counts with AO revealed that total bacterial numbers ranged from 10^5 to 10^6 cells $\cdot$ ml^{-1} (see samples in Table 6). When total counts were compared with the mean numbers of CFU on DP and SMA (by using the SPC technique), DP usually recovered only a small percentage of the total bacteria, but a greater percentage than did SMA (Table 6). It is also interesting to note that the direct counts of all but one of the chlorinated samples were reduced in comparison with those of samples from the point preceding chlorination (K3 and Res. K3 had the same count on 11/12/81).

DISCUSSION

Our findings that more organically dilute media recover higher numbers of heterotrophic bacteria from chlorinated water agree with other recent findings for potable water (6, 15, 21). Of the four media used in the enumeration studies, DP usually recovered the highest numbers of CFU (Tables 3 and 4 and Fig. 2). Because DP was the most dilute in terms of organic carbon (Table 2), the results suggest either that more species of bacteria in chlorinated water from the Seattle water distribution system can grow on this medium or that damage that has occurred during chlorination can be more readily repaired when bacteria that are damaged grow on this medium. Further experiments beyond the scope of this study would be necessary to determine which possibility is correct. However, reports have been made that a number of species of bacteria are able to grow in tap water with low concentrations of organic substrates (21–24). These reports, combined with the demonstration of oligotrophic bacteria from other aquatic environments (14), argue for the first alternative. Furthermore, we know that one species of *Hyphomonas* grew only on DP.

The spread plates of DP, CPS, and usually R2A recovered much higher numbers of CFU than did the pour plates of SMA (SPC procedure) in the current study, and this result is

TABLE 5. Diversity indexes of source water (CPR1) and chlorinated water samples collected on 9/2/81 and counted after 28 days

Sample	No. of bacteria/ml	Diversity index[a]	Raw data[b]
CPR1	3,600	4.14	9 (2), 4 (3), 2 (4), 1 (17)
I3	210	1.85	27, 13, 4, 3, 2 (2), 1 (4)
K3	1,700	2.81	21, 6 (4), 2 (4), 1 (2)
L3	2,900	1.23	37, 9 (2)
M3	150,000	1.08	30, 5, 3, 1 (2)

[a] Based upon 55 colonies, except for M3, for which 40 colonies were used. CPR1 diversity was determined with CPS, whereas all chlorinated water sample diversities were determined with DP.

[b] Number of colonies of each colony type. For example, for CPR1 2 colony types (i.e., strains) were represented by 9 colonies each, 3 strains were represented by 4 colonies each, 4 strains were represented by 2 colonies each, and 17 strains were represented by unique colonies, giving a total of 55 colonies.

TABLE 6. Comparison of direct AO microscopic counts with viable counts for samples from the Cedar River watershed

Date	Sample	Residual chlorine (mg · liter^{-1})	No. of bacteria/ml counted by or on[a]: AO (SE)	DP (SD)	SPC (SD)	% Viable DP/AO	% Viable SPC/AO
9/2/81	CPR1	ND[b]	8.6×10^5 (2.4×10^4)	3.6×10^3 [1]	ND	0.42	
	CPT0	ND	4.2×10^5 (2.7×10^4)	1.2×10^4 [1]	ND	2.86	
	LY	ND	8.5×10^5 (5.7×10^4)	1.0×10^5 (2.4×10^4)	ND	11.8	
	CLT2	ND	8.1×10^5 (2.1×10^4)	6.0×10^1 [1]	ND	0.019	
	Res. L3	ND	5.3×10^5 (1.8×10^4)	1.6×10^5 (2.4×10^4)	7.4×10^1 [1]	30.2	0.014
	L3	ND	3.5×10^5 (1.3×10^4)	3.1×10^3 (2.0×10^2)	ND	0.89	
	Res. M3	ND	1.2×10^6 (1.7×10^4)	4.0×10^5 (3.6×10^4) [2]	6.4×10^1 [1]	33.6	0.0054
	M3	ND	5.9×10^5 (1.4×10^4)	1.6×10^5 (9.2×10^3) [2]	$<1.0 \times 10^0$ [1]	27.1	<0.00017
	Res. I3	ND	2.8×10^5 (5.9×10^4)	1.1×10^3 (1.1×10^2)	5.8×10^1 [1]	0.39	0.21
	I3	ND	2.2×10^5 (1.9×10^4)	5.1×10^2 (9.3×10^1)	$<1.0 \times 10^0$ [1]	0.23	<0.00046
	Res. K3	ND	2.1×10^5 (4.0×10^4)	8.9×10^4 (1.2×10^4)	1.0×10^2 [1]	42.4	0.48
	K3	ND	1.8×10^5 (2.5×10^4)	1.7×10^3 (2.6×10^2)	$<1.0 \times 10^0$ [1]	0.94	<0.00056
11/1/81	CRP1	ND	1.3×10^6 (1.4×10^4)	1.4×10^4 (1.1×10^3)	2.6×10^1 (1.2×10^1)	1.08	0.0020
	CPT0	ND	7.6×10^5 (3.6×10^4)	9.8×10^1 [1]	3.0×10^0 (3.0×10^0)	0.013	0.00049
	LY	ND	ND ND	ND ND	ND ND	ND	ND
	CLT2	ND	6.0×10^5 (3.5×10^4)	1.9×10^1 (7.2×10^0) [2]	0.3×10^0 (0.6×10^0)	0.0032	0.000050
	Res. L3	ND	9.1×10^5 (4.8×10^4)	$<0.3 \times 10^1$	0.3×10^0 (0.6×10^0)	<0.033	0.000033
	L3	0.60	8.2×10^5 (4.3×10^4)	$<0.3 \times 10^1$	$<0.3 \times 10^0$	<0.0034	<0.000037
	Res. M3	ND	1.2×10^6 (7.9×10^4)	4.6×10^5 (2.1×10^4)	2.3×10^0 (2.5×10^0)	38.3	0.00020
	M3	0.62	1.1×10^6 (7.4×10^4)	1.6×10^5 (1.1×10^4)	1.3×10^0 (1.2×10^0)	14.5	0.00012
	Res. I3	ND	1.2×10^6 (5.3×10^4)	1.5×10^2 (5.2×10^1)	$<0.3 \times 10^0$	0.41	<0.000025
	I3	0.75	9.6×10^5 (5.7×10^4)	5.0×10^0 (1.4×10^0) [2]	2.0×10^0 (1.8×10^0)	0.00052	0.00021
	Res. K3	ND	1.3×10^6 (1.8×10^5)	3.4×10^3 (1.8×10^3)	1.3×10^0 (1.6×10^0)	0.026	0.00010
	K3	0.50	1.3×10^6 (9.1×10^4)	7.0×10^1 (4.3×10^1) [2]	$<0.3 \times 10^0$	0.0054	<0.000023

[a] Three plates were usually used for each viable enumeration; if fewer than three plates were available (due to overgrowth or other problems), the number used is shown in brackets.
[b] ND, Not determined.

consistent with those of other recent reports (6, 15, 20). Furthermore, the SPC procedure does not provide a proportionately lower count from sample to sample and often fails to recover any isolates (Table 4). The lower numbers of CFU recovered by the SPC procedure is most likely due to the thermal sensitivity of the bacteria (4) as well as the organic richness of the medium. The low amount of organic matter probably also contributed to the overall higher recovery on DP than on CPS. The most recent issue of *Standard Methods for the Examination of Water and Wastewater* (1) describes a spread plate method that uses R2A for enumerating heterotrophs.

It was previously shown for bacteria from the Seattle water distribution system that incubation temperatures of 35°C and higher were not as favorable for the maximum recovery of CFU as 20°C (6). However, it was not known whether an incubation temperature of 30°C was also too high for maximum recovery. This was an important question to answer because of the prolonged period of incubation needed to provide highest recoveries at 20°C (28 to 30 days). We decided also to test temperatures of 11 and 30°C and a temperature shift from 20 to 30°C. The hypothesis behind the temperature shift experiment was that some bacteria might be able to grow at 30°C but could not survive a rapid shift to 30°C at the time of plating, when the environmental temperature was 10 to 20°C. Statistically significant results occurred with M3 and I3 samples. In both cases 20°C was the optimum incubation temperature for both DP and CPS, confirming the results of Fiksdal et al. (6) and agreeing with the results of Reasoner and Geldreich (15) for R2A. The lack of a significant difference between the different incubation temperatures for the other chlorinated samples may have been due to unusually high levels of residual chlorine.

The major drawback of using DP, although it consistently recovers high numbers of heterotrophic bacteria from chlorinated water, is the length of incubation required at 20°C. This extensive time makes the medium prohibitive for routine monitoring of distribution systems. Nonetheless, it is recommended for researchers who wish to recover the predominant heterotrophs and study the important species in chlorinated water. Furthermore, our results indicate the need to develop a more rapid methodology than viable plate counting for monitoring the total viable heterotrophs in distribution systems.

Our inability to show positive or negative relationships between the mean numbers of CFU recovered and the physical and chemical parameters measured is not really surprising. Jones (12) used a principal component analysis and multiple regression analyses to investigate the effects of environmental factors on estimated viable populations of planktonic bacteria in lakes and experimental enclosures, found that the variation in data could be explained by five regressor values, and listed a number of others that may add further information. In short, attempts to understand the role of environmental factors in the recovery of heterotrophic bacteria from chlorinated systems would require measurements of additional parameters besides temperature, pH, and residual chlorine. In contrast, it is interesting to note the apparent direct relationship between a decrease in total AO counts and chlorination.

The diversity data obtained in this investigation were consistent with preliminary data previously reported for the Seattle drinking water system (6) as well as other surveys we have analyzed (data not shown). The operation of this drinking water treatment and distribution system evidently results in the selection of a limited number of bacterial species that predominate in the reservoirs and are ultimately found in the final chlorinated samples. It is interesting to

note that many of the surviving bacteria were pigmented. Reasoner and Geldreich (15) have also reported high proportions of pigmented bacteria in chlorinated treatment waters. The observation that the reduction in diversity occurred in Lake Youngs, the reservoir following the first chlorination, suggests either of two possibilities: either the pigmented forms in these samples are more chlorine resistant than most bacteria from source waters and are therefore selected for by chlorination, survive in the reservoirs, and grow or they are not more chlorine resistant, but the physical, chemical, and biological conditions in the open reservoirs favor their growth, and they have become established over a long period of time. Additional studies would be needed to determine which of these alternatives is true. The determination of which alternative is correct is important, as chlorine-resistant bacteria may also be antibiotic (2, 3)- and metal (5)-resistant, providing a potential reservoir of resistance factors for nonresistant pathogenic bacteria.

With respect to the direct count data, it is interesting to note that the percentages of recovery of viable heterotrophs in the reservoir samples were often high, which is characteristic of eutrophic waters (up to 40% was reported when DP was used). In contrast, source waters (CPR1) always had recoveries of less than 1%, typical of oligotrophic and mesotrophic aquatic habitats in the Pacific Northwest (19). Thus, in this system, the process of water treatment often produces an apparent eutrophication effect, at least with respect to the heterotrophic bacterial community. This effect may also be related to the changes in diversity that were observed. The apparent eutrophication effect may be due to the release of organic nutrients from algae and from bacterial and other microbial biomasses by chlorination, making this material available to planktonic bacteria in the downstream reservoirs.

Recommendations. (i) Choice of medium. SMA was inferior to the other media tested in this investigation. We recommend interim adoption of R2A as the medium of choice for chlorinated drinking waters. CPS, which is similar in composition to R2A, appears to be comparable to R2A and could be used in place of it (6). The best medium for Seattle's chlorinated drinking waters was the most dilute medium used, DP. Further testing should be done in other regions to determine whether it is as effective as it is in Seattle.

(ii) Plating of viable samples. Pour plating should be abandoned because it frequently causes death of heterotrophic bacteria from chlorinated samples due to thermal shock. Spread plating is the preferred method for enumerating heterotrophic bacteria from drinking waters. Although membrane filtration procedures have been used with success by some, we found that they did not provide as high counts as did spread plating when samples of Seattle drinking water were analyzed (6). Thus, spread plating should be adopted unless studies have shown it to be less satisfactory than membrane filtration procedures or other techniques for a particular sample.

(iii) Plate size. Large petri dishes (150-mm diameter) should be used for samples containing low concentrations of bacteria, as they allow one to place a greater volume of the sample on the plate (the surface area is ca. 2.3 times greater than that of regular petri dishes [100-mm diameter]). With proper drying at least 1.0 ml of a sample can be absorbed on the medium in a large petri dish.

(iv) Temperature of incubation. Incubation temperatures for the Seattle drinking water samples yielded lower counts when they exceeded the ambient water temperatures by 17°C. For example, the data shown for M3 samples in Fig. 3A were obtained when the water temperature was 13°C. For this system, it is undesirable to handle and incubate samples at temperatures above 20°C. As a general rule, we recommend that incubation temperatures for viable heterotrophs should be no greater than 10°C above the temperature of the sampling water if one wishes to obtain a maximum recovery of bacteria. Of course, when lower temperatures are used, it is necessary to incubate longer for maximum recovery. We recommend that 28 to 30 days be used for 20°C.

(v) Biomass procedures. It is evident that, from a public health perspective, current and prospective viable plating procedures cannot provide satisfactory estimates of viable bacteria in water treatment and distribution systems. The best available procedures require incubation periods that are excessive for the rapid monitoring of drinking water quality. Thus, it is imperative that alternative microbial biomass procedures, based perhaps on the quantification of ATP or tetrazolium dye reduction in conjunction with direct microscopic counting with AO or similarly sensitive techniques, be developed to provide rapid estimates of bacterial concentrations in drinking waters.

(vi) Effect of chlorination on diversity. Additional studies should be conducted to determine whether the reduction in diversity that we observed in these studies is due solely to chlorination or whether other factors in the treatment process are in part responsible. If chlorination is responsible for the reduction in diversity and the selection of pigmented bacteria, it may be possible to modify the treatment process to make these bacteria more susceptible to disinfection.

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